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Mosaic Disease of Tobacco

Action of Proteoclastic Enzymes on the Virus Fraction
Nature of the Virus Fraction from Various Species of Plants

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Mosaic Disease of Tobacco

Action of Proteoclastic Enzymes on the Virus Fraction
Nature of the Virus Fraction from Various Species of Plants

A. Frank Ross and C. G. Vinson

Previous work[19, 20, 21, 22]* has indicated that the virus of mosaic disease of tobacco is proteinaceous in character. If the virus is a protein, then certain enzymes should inactivate it. In any event it might be possible to effect purification of the virus by digesting away some of the accompanying material, in case the fractions were not absolutely pure.

Accordingly the effect of trypsin on the tobacco mosaic virus under different experimental conditions was investigated. Several types of purified preparations were used in view of the possibility that some might be more favorable to enzyme attack than others. The preparations used included the decomposed safranin precipitate, the decomposed second safranin precipitate, the neutral phosphate eluate of the lead precipitate, the dilute sodium carbonate eluate of the lead precipitate from the decomposed safranin precipitate, the dilute phosphate eluate of the lead precipitate from cleared juice, and the decomposed safranin precipitate from the phosphate eluate of the lead precipitate from cleared juice. In some experiments the preparations were heated to 70°C. for 20 minutes, then cooled before addition of the enzyme. The heating was done in an attempt to destroy any heat unstable antienzymes that might be present.

Commercial trypsin was added to the preparations at the rate of 2 mg. per cc. The controls contained an equal amount of tryspin that had been inactivated by heating in the presence of water at 100°C. for 20 minutes. Toluene and chloroform were added as preservatives[1].

The infectivity of a preparation or digest was determined by inoculation into young potted tobacco (*Nicotiana Tabacum,* var. Turkish) by the pin prick method[7]. In preliminary experiments, sets of ten plants were used, four leaves on each plant being punctured. When a more accurate estimation of infectivity was desired, sets of one hundred plants were inoculated, puncturing one leaf per plant. Inoculations were made both with and without charcoal.

*Numerical references are to "Bibliography," page 18.

TABLE 1.—RESULTS OBTAINED ON INCUBATING PURIFIED VIRUS PREPARATIONS WITH TRYPSIN.

Experiment		Results of inoculating with the digests and control at the end of the digestion period					
		Digest I		Digest II		Control	
Date	Digestion Period Days	Wihtout Charcoal	With Charcoal	Without Charcoal	With Charcoal	Without Charcoal	With Charcoal
4/ 1/33	3	2	--	3	--	6	--
4/ 3/33	3	5	--	5	--	10	--
4/14/33	14	2	4	1	3	9	10
4/29/33	14	5	5	3	6	9	10
6/24/33	26	0	0	0	2	7	9
6/29/33	30	3	7	4	8	10	10
10/14/33	7*	2	5	3	5	9	10
10/21/33	6*	3	8	3	5	10	10

Experiment		Results of inocultaing with digests and control at end of digestion period after they had been heated for 20 minutes at 68-70° C					
		Digest I		Digest II		Control	
Date	Digestion Period Days	Without Charcoal	With Charcoal	Without Charcoal	With Charcoal	Without Charcoal	With Charcoal
4/ 1/33	3	5	--	7	--	8	--
4/ 3/33	3	5	--	5	--	8	--
4/14/33	14	6	8	6	9	9	10
4/29/33	14	9	8	8	8	8	10
6/24/33	26	7	4	2	5	7	8
6/29/33	30	4	9	7	9	10	9
10/14/33	7*	8	10	7	9	10	10
10/21/33	6*	4	7	5	5	10	10

*Trypsin + enterokinase.

In each case the infectivity of a preparation was determined before the addition of trypsin. After incubation the digests were used for inoculation then heated to 70°C. for 20 minutes and again used for inoculation. The digests were kept near 70°C. until the inoculation was completed, then cooled before the addition of charcoal. The controls were heated in exactly the same manner.

Incubation with trypsin in all cases resulted in a great decrease in infectivity. Inactivation occurred within an hour after addition of the trypsin. Incubation at lower temperatures also resulted in inactivation. Upon prolonged incubation, the controls decreased a little in infectivity but very much less than the digests.

Heating the digests to 70°C. for 20 minutes resulted in a great increase in infectivity. As a rule, they did not become as infectious as the controls, which were also heated. This would seem to indicate that the greater part of the inactivation is due to adsorption but does not exclude the possibility of some permanent inactivation due to the trypsin. Provided the inactivation was due to adsorption, the failure of the digests to become as infectious as the controls might be due to an incomplete release of the virus. Attempts were made to heat the digests to higher temperatures, but these attempts invariably resulted

in the inactivation of the virus, inactivation being practically complete at 80°C. for 20 minutes.

The differences between the infectivity of the controls and the digests after heating became more pronounced as the incubation period was lengthened.

This indication of the formation of a virus-trypsin combination[3] suggested the possibility of proteolysis taking place under the proper conditions. The preliminary step in many enzyme reactions is the formation of an intermediate product between the enzyme and substrate[17 12].

The pH of the preparations used in the foregoing experiments varied from 6.2 to 7.0. Trypsin shows activity at these reactions but its maximum activity is at pH8 for most substrates[24]. Several experiments were conducted using preparations buffered at pH8. The results resembled in most respects those of the previous experiments, except that the stability of the controls was lessened.

The addition of enterokinase[25]* to the trypsin-virus digests did not cause permanent inactivation. Calcium sulfate[4], calcium chloride[9], barium chloride and ammonium sulfate[10] have been reported as activators of trypsin. The addition of these salts, both alone and in combination with enterokinase, to trypsin digests did not cause permanent inactivation of the virus.

A mixture of pepsin and trypsin was less effective in inactivation of the virus than trypsin alone. This might possibly be due to the digestion of trypsin by pepsin[18]. The combination of enzymes did not cause permanent inactivation. Increasing the concentration of trypsin in the digests from two to twenty milligrams per cc apparently did not cause a further decrease in activity.

PURIFICATION OF FRACTIONS BY MEANS OF ENZYMES

Since tryptic digestion was found to have little or no permanent effect on the virus, it was deemed possible to use trypsin in the purification of the virus.

Portions of the decomposed safranin precipitate were incubated with trypsin. After remaining in the incubator for several days, the digests were heated to 70°C. for 20 minutes, cooled quickly, centrifuged and safranin added. The resultant safranin-virus precipitate was washed, then decomposed with Lloyd's reagent. The supernatant liquid was frozen, thawed, and centrifuged. The virus was precipitated by N/1 aluminum sulfate. The precipitate was used for total solids,

*Enterokinase, the natural activator of trypsin, was prepared from calf intestine according to the method of Waldschmidt-Leitz.

ash, nitrogen, and phosphorus determinations. Controls for these determniations consisted of similar determinations made on the aluminum sulfate precipitate prepared in exactly the same manner except that the digestion with trypsin was omitted. Inoculations were made with all fractions, using sets of 100 plants.

The infectivity, as well as the amount of solids, ash, and nitrogen was less in the case of the fraction obtained from the digest, than in that from the control. However, there was no significant difference in the percentage of nitrogen in the two precipitates.

It was thought possible that a portion of the nitrogen found in the fraction recovered from the digests was due to some trypsin being carried along in the procedure[14]. An aqueous suspension of trypsin was therefore precipitated with safranin, using the same concentration of each as used in the digests. Both active and inactivated trypsin were used. The safranin-trypsin precipitate was decomposed with Lloyd's reagent, and the resulting supernatant liquid treated with N/1 aluminum sulfate. A slight amount of precipitate was formed but it contained no Kjeldahl nitrogen.

It seems then, that the protein found in the virus preparations is not attacked to any great extent by trypsin.

The action of papain on the virus was investigated, using the same general procedures used with trypsin. It was found that papain inactivated the virus to a marked degree[13]. There was no significant increase in infectivity after heating the digests to 70°C. Papain is not inactivated at 70°C., but a higher temperature could not be used as it would result in inactivation of the virus. When KCN, an activator or papain, was added with the papain, complete inactivation occurred in a shorter length of time than when no KCN was added.

If the inactivation was of the nature of an adsorption, it would be reasonable to believe that an increase in temperature would result in less adsorption. No such decrease was observed at higher temperatures. These observations indicate that the inactivation of the virus is more complex than that of simple adsorption.

USE OF MICROORGANISMS IN PURIFICATION OF VIRUS FRACTIONS.

After commercial enzyme preparations were found to give little promise of use in further purification of virus fractions, attention was turned to the enzymes produced by microorganisms. The first step was to determine whether or not the virus is resistant to attack by microorganisms.

The effect of mixed cultures of microorganisms was first determined. Purified virus preparations were allowed to stand uncovered

in the laboratory for several hours, then incubated without presrevatives at 37°C. When the decomposed safranin precipitate was used, the digests became cloudy and after a few days a precipitate began to settle out. A distinct putrid odor developed that became less noticeable in a week or 10 days, giving way to a somewhat less offensive odor. In the case of the phosphate eluate of the lead precipitate, the digests became cloudy and gave a slight precipitate but a less offensive odor was produced. The digests became more basic but never more so than pH8. Controls consisted of similar preparations to which toluene and chloroform had been added.

A decrease in infectivity occurred in both the digests and in the controls. The decrease occurring in the digests was appreciably greater than that occurring in the controls. The rate of inactivation was slow, however, being by no means complete in five and one-half months. Cultures of *B. proteus* and *B. aerogenes* were no more effective in inactivating the virus than the mixed culture.

The inactivation of the virus by bacteria does not eliminate their use in virus purification, as inactivation occurs slowly and does not go to completion in moderate length of time. A good yield of virus would be desirable, but certainly not essential.

Portions of the purified virus preparations were allowed to stand exposed to the laboratory air for several hours. In some cases small portions of previous digests were added. The preparations were incubated at 37°C. without preservatives.

After an incubation period of several days the digests were removed, frozen, then thawed and centrifuged to remove much of the precipitate that had formed. Sanfranin was then added. The virus-safranin precipitate was decomposed with Lloyd's reagent and the resultant supernatant liquid frozen, thawed, then centrifuged and filtered through a Berkefeld W filter. The virus was then precipitated with $N/_1$ aluminum sulfate. The precipitate obtained upon centrifuging was washed, then analyzed for nitrogen, phosphorus, total solids, and ash. Inoculations were made with all fractions, using sets of 100 plants each.

For controls, portions of the same preparations used for incubation were treated in exactly the same maner; except that they were not incubated, or, as in some cases, incubated with toluene and chloroform added as preservatives.

Fractions obtained from the digests were not in general as infectious as those from the controls.

The aluminum sulfate precipitate obtained from the digests contained smaller amounts of organic material, ash, phosphorus, and ni-

trogen than the corresponding precipitate from the controls. The percentages of nitrogen, ash, and phosphorus, calculated on the basis of total solid content were approximately the same in the two precipitates.

ACTION OF SOIL MICROORGANISMS ON THE VIRUS

The action of soil microorganisms was also investigated. Small quantities of soil suspensions were added to purified virus preparations, and the preparation incubated without preservatives. Controls were prepared in the same manner and toluene and chloroform were added as preservatives. A second control consisted of a portion of the preparation to which no soil was added and incubated without preservatives. Nessler's tests were made on the preparations before and after incubation.

A decrease in infectivity occurred in the digests and in the controls. The greater inactivation occurred in the digests but they were only slightly less infectious than the control containing no soil and no preservative. Those containing the preservatives were less infectious than the preparation before incubation, but more infectious than the digests.

Inactivation was associated with formation of ammonia. The preparations before incubation gave a very faint or no color with Nessler's reagent. The digests and controls incubated without preservatives gave distinct coloration, the intensity being roughly proportional to the inactivation that had occurred. The controls to which toluene and chloroform had been added usually gave a faint color. A slight decrease in hydrogen-ion concentration occurred but the digests were never more basic than pH8.

The data indicate that the virus is slowly inactivated by microorganisms.

THE NATURE OF THE ACTION OF TRYPSIN ON THE VIRUS

Caldwell[5] found that after incubating the virus of aucuba mosaic of the tomato with trypsin, infectivity could be restored by heating the digests to 70°C., the inactivation temperature of trysin. Stanley[15] found that trypsin digests of the tobacco mosaic virus behaved in the same manner. Stanley concluded that the inactivation was due to the effect of the enzyme upon the host plant.

Stanley used *Nicotiana glutinosa* and *Phaseolus vulgaris* as host plants and used the local lesion method of count. It was thought desirable to determine whether or not the effects noted by Stanley would be apparent, using *N. Tabacum.*

EXPERIMENTAL

Portions of purified virus preparations were treated with trypsin (50 mg. per 25 cc.) and used for inoculation as were the virus preparations themselves. Ten plants were inoculated in each case, using five pins and puncturing four leaves per plant. Leaves of tobacco plants were rubbed with a suspension of trypsin in water of the same concentration as above and allowed to dry 6 hours. The plants were then inoculated with the original virus preparations by the pin prick method of inoculation. Still other plants were inoculated with the trypsin suspension by the pin prick method. After allowing to dry 6 hours, the same plants were used for inoculation with the original virus preparation, using the same pins. Care was taken to insert the pins in the holes made in the inoculation with the trypsin suspension. Inoculations were made with and without charcoal.

In some cases, the virus preparation, the virus-trypsin digests, and the trypsin suspension were kept in an incubator at 37°C. for some time, using toluene as preservative. This was done in order to duplicate as nearly as possible the conditions obtained when inoculations were made in incubation experiments.

The purified virus preparations used were the decomposed second safranin precipitate described by Vinson[22] and phosphate eluate of the lead precipitate from cleared juice described by Vinson and Petre[21].

TABLE 2.—PLANTS DISEASED.
(10 Plants Inoculated)

Treatment	Decomposed		PO_4 eluate		PO_4 eluate inc. 14 da.		PO_4 eluate inc. 5 da.		PO_4 eluate inc. 1 day		Average	
	Saf. −c	Ppt. +c	−c	+c	−c	+c	−c	+c	−c	+c	−c	+c
Original Virus Preparation	10	10	10	10	3	10	7	8	10	10	8	9.6
50 Mgs. Trypsin per 25 cc. Virus Preparation	3	7	7	9	0	1	4	8	1	5	3	6
Leaves rubbed with trypsin, then inoculated with prep.	8	10	7	10	9	10	6	7	6	5	7.2	8.4
Inoculated with trypsin, then with virus prep.	8	9	8	9	10	10	7	9	2	6	7.0	8.6

The data indicate that trypsin does not inactivate the virus when applied to the leaves before inoculation as much as when it is suspended in the virus preparation. Hence inactivation is greatest under conditions most favorable to adsorption. One would expect some virus to come in contact with the enzyme on the leaves and be adsorbed. Some decrease in infectivity is apparent. If the inactivation were due to action of the enzyme on the leaf, the greatest inactivating action would surely be noted when the enzyme had been applied to the leaf and had had ample chance to exert its action.

Pepsin

Lojkin and Vinson[11] reported that the tobacco mosaic virus was not inactivated by pepsin at pH7. At pH3 inactivation of the controls occurred. Stanley[16] using *N. glutinosa* and *Phaseolus vulgaris* as host plants, found that pepsin inactivated the virus at pH3 and that the controls remained infectious. He suggested that the apparent inactivation of the controls used by Lojkin and Vinson might have been due to the effect of the high hydrogen-ion concentration on the host plant, *N. Tabacum,* used by them.

To determine if this were true, experiments were conducted in which the pH of the digests was changed after incubation to a range that has no effect on the host plant.

The dilute phosphate eluate of the lead precipitate from cleared juice described by Vinson and Petre[21] was prepared. 2N HCl was added to the buffered preparation drop by drop with constant stirring until a pH of around 3 was obtained. Inoculations were made both before and after the addition of the acid. A white flocculent precipitate was formed. After mixing thoroughly, 25 cc. was pipetted into each of three flasks. To Nos. I and II, 50 mgs. commercial pepsin was added (in 0.1 N HCl). To the check, 50 mgs. of pepsin that had been inactivated by heating at 100°C. for 20 minutes was added. Toluene and chloroform were added as preservatives, the flasks stoppered and placed in the incubator at 37°C. Several days later, the flasks were removed and 5 cc. of the contents removed from each flask, diluted with 5 cc. redistilled water and used for inoculation. Five cc. portions from each flask were also diluted with 5 cc. M/3 mixed phosphate of pH7 and used for inoculation. The pH of the latter mixture was always between 6.5 and 7. Ten plants were used for inoculation, four leaves of each being punctured with 5 pins. Inoculations were made with and without charcoal.

It is evident from Table 3 that pepsin inactivates the virus. The controls remained infectious even when inoculated into the plant at pH3. Increasing the pH seemed to have little or no effect on infectivity. Heating the digests either at pH3 or after being brought to pH7 failed to increase their activity.

It was thought possible that the inactivation might have been due to certain products formed by hydrolysis of other substances present. In investigating this possibility, 50 cc. portions of freshly prepared virus preparations were buffered at pH7, added to the digests, and the flasks returned to the incubator. The pH of the resulting mixture was always between 6.5 and 7. Pepsin is inactive

TABLE 3.—EFFECT OF INCUBATING THE VIRUS WITH PEPSIN

pH	Days in Incubator	Original Preparation		Preparation +HCl			Digests		Digests + PO_4 Buffer	
		−c	+c	−c	+c		−c	+c	−c	+c
2.88	27	8	10	10	10	I	2	1	0	1
						II	0	1	1	2
						ck.	10	8	9	9
3.02	26	6	7	10	10	I	1	1	1	1
						II	1	0	0	1
						ck.	9	10	10	10
3.04	31	7	10	8	9	I	0	0	0	0
						II	0	0	0	0
						ck.	5	10	7	10
3.05	44	9	10	10	10	I	0	0	0	0
						II	0	1	0	0
						ck.	8	9	8	10
3.1	46	9	10	10	10	I	4	2	0	0
						II	0	0	0	0
						ck.	5	8	5	8

+c = with charcoal. −c = without charcoal. 10 plants inoculated.

TABLE 4.—EFFECT OF DECOMPOSITION PRODUCTS OF PEPTIC HYDROLYSIS ON THE VIRUS.

pH of Digests	Days in Incubator	Digests				Digests + fresh virus preparation left in incubator for									
		At pH3		+PO_4		5 days		13 days		21 days		28 days		50 days	
		−c	+c	−c	+c	−c	+c	−c	+c	−c	+c	−c	+c	−c	+c
3.05	I	4	2	0	0	8	10	--	--	10	10	--	--	7	10
	44 II	0	0	0	0	10	10	--	--	10	10	--	--	10	9
	ck.	5	8	5	8	10	10	--	--	9	10	--	--	10	10
3.04	I	0	0	0	0	--	--	8	8	--	--	7	7	--	--
	31 II	0	0	0	0	--	--	7	10	--	--	7	10	--	--
	ck.	5	10	7	10	--	--	9	9	--	--	9	9	--	--

+c = with charcoal. −c = without charcoal. Numbers refer to number of plants diseased out of 10 inoculated.

at that pH. After several days in the incubator, the flasks were removed and the contents used for inoculation.

Little or no further inactivation occurred. It is possible that any such compounds, if formed might be toxic in acid media only. It would then be difficult to differentiate between such an effect and peptic activity. Destruction of the enzyme by heat or other means might also destroy the toxic compound. Stanley[16] found little or no further inactivation at pH3.

Two experiments were conducted to determine the approximate rate of proteolysis. Samples were prepared as previously described and placed in the incubator. The digests were removed at intervals and portions of each used for inoculation, then replaced in the incubator.

Pepsin inactivated the virus slowly, causing a gradual reduction in infectivity. Inactivation was practically complete at the end

of 7 days. Addition of phosphate buffer or heating did not increase the infectivity of the digest incubated 14 days.

Investigation of Purified Virus Preparations From Different Host Plants

Nearly all investigators have used diseased Turkish tobacco plants as a source of the tobacco mosaic virus. The virus has a wide host range as shown by Holmes[6] and by Grant[8]. Many of these differ widely from Turkish tobacco and would be expected to yield juice differing in composition from that of Turkish tobacco. If extraneous material is carried along with the virus through the purification procedures when Turkish tobacco juice is used, such substance or substances might not be present in juices of other species of plants. Use of other plants as a source of the virus might, therefore, result in further purification of the virus.

TABLE 5.—RATE OF INACTIVATION OF THE VIRUS BY PEPSIN.

	Plants Diseased (10 plants inoculated)													
	Preparation		Preparation +HCl		Digests after being at 37° C. for:									
					1 hr.		1 day		2 days		4 days		6 days	
pH	−c	+c	−c	+c	−c	+c	−c	+c	−c	+c	−c	+c	−c	+c
				I	10	10	9	10	--	--	6	8	--	--
3.03	10	10	9	10 II	9	10	9	10	--	--	7	9	--	--
				ck.	10	10	10	10	--	--	10	10	--	--
				I	9	9	--	--	9	8	5	9	2	6
3.0	10	10	10	10 II	9	10	--	--	9	10	9	7	3	7
				ck.	9	10	--	--	9	10	10	10	10	10

	Plants Diseased (10 plants inoculated)													
	Preparation		Preparation +HCl		Digests after being at 37° C. for:									
					7 days		8 days		10 days		12 days		14 days	
pH	−c	+c	−c	+c	−c	+c	−c	+c	−c	+c	−c	+c	−c	+c
				I	0	0	--	--	--	--	--	--	--	--
3.03	10	10	9	10 II	0	4	--	--	--	--	--	--	--	--
				ck.	6	8	--	--	--	--	--	--	--	--
				I	--	--	0	1	4	6	3	2	0	4
3.0	10	10	10	10 II	--	--	0	4	2	3	2	4	1	1
				ck.	--	--	9	10	10	9	9	10	10	10

The possibility of obtaining nitrogen free preparations from other species of plants was suggested in part by Barton-Wright and McBain's[2] report of obtaining nitrogen free preparations from *Nicotiana macrophylla*. They combined two procedures worked out by Vinson and Petre[20, 21]. Vinson[23] later repeated the procedures, using *N. Tabacum* and found nitrogen to be present.

Diseased plants of *N. macrophylla* were grown in a greenhouse. When of good size they were cut, frozen, thawed, and the juice

expressed by a hydraulic press. The procedure used by Barton-Wright and McBain was followed in detail. It consisted essentially of clearing the juice with basic lead acetate, precipitation of the virus with neutral lead acetate followed by treating the precipitate with M/3 KH_2PO_4, then with water and elution of the virus with phosphate buffer of pH7. The virus was precipitated by aqueous safranin and the safranin virus precipitate decomposed with amyl alcohol. Care was taken in each extraction to retain the material held in the interfacial layer. It is probable that this material contains much of the virus. Nitrogen analyses (Kjeldahl) were run on the resulting preparations. Liter samples were used for analysis after being concentrated. Inoculations were made with the original juice and with the preparations.

Nitrogen was present in all cases. The amount of nitrogen was much less than that in preparations obtained by other methods. However, the infectivity was correspondingly low. If smaller samples had been used the results of analyses would have been doubtful.

TABLE 6.—NITROGEN CONTENT AND INFECTIVITY OF PREPARATIONS FROM N. MACROPHYLLA, USING AMYL ALCOHOL TO DECOMPOSE THE VIRUS-SAFRANIN PRECIPITATE.

Trial	Original Juice			Final Preparation			
	Plants Inoc.	Plants Diseased		Plants Inoc.	Plants Diseased		Mgs. N per liter
		−c	+c		−c	+c	
1	10 100	8 46	10 89	10 100	3 7	8 35	13.3
2	10 100	7 44	9 85	10 100	8 36	10 40	4.6
3	--	--	--	--	--	--	3.7

In the investigations of other species, a different method of procedure was used. A liter of the juice was treated with 100 cc. neutral lead acetate (22 gm. per L.). The precipitate was washed twice with about 1200 cc. M/3 KH_2PO_4 and then suspended in about 400 cc. water and 200 cc. phosphate buffer of pH7. After standing one hour, the precipitate was centrifuged off and to the supernatant liquid 400 cc of 1 per cent aqueous safranin solution was added. After mixing, it was allowed to stand until the precipitate had settled. Most of the supernatant liquid was siphoned off and the remaining suspended precipitate divided equally into four centrifuge tubes and centifuged. Each precipitate was then suspended in 90 cc. of water, disintegrated thoroughly, then decomposed with Lloyd's reagent as described by Vinson[22]. A gram of the reagent

was usually added for each cc. of the precipitate obtained after 15 minutes centrifuging in pointed tubes.

The supernatant liquids were frozen, thawed, centrifuged, then extracted with N-amyl alcohol until free of red. They were again frozen, thawed, and centrifuged. N/1 acetic acid was then added until a precipitate was obtained. The precipitate was centrifuged off, washed, then used for analysis. In cases where solids and ash determinations were made, the extracted supernatant liquids were diluted to 400 cc. and four portions of 100 cc. each pipetted into each of four tubes before precipitation with acid. Two of the precipitates were transferred quantitatively to weighed silica dishes and the other two to Kjeldahl flasks. This procedure gave excellent preparations. The precipitates were usually colorless but occasionally were slightly brown or amber. The preliminary clearing with basic lead acetate was omitted in order to retain as much of the virus as possible. Preliminary clearing with the basic lead acetate was found to have little or no effect on the appearance and composition of the final product.

In all previous procedures, making use of Lloyd's reagent, much of the reagent remained in suspension and could not be removed by centrifuging. In so doing it retained some of the dye and probably other substances as well. The ash content was always very high. It was found that the amyl alcohol not only removed the safranin but also the suspended Lloyd's reagent, some pigment and possibly other extraneous material. Usually two or three extractions were sufficient. The alcohol apparently removed or destroyed none of the virus. It is believed that this procedure gives a product that is as pure as or purer than any that has been described previously.

Enough material was available in the case of some species to make solids and ash determinations as well as nitrogen determinations. With others only nitrogen determinations were made.

Inoculations were made with all fractions. In the tables only the results of inoculations with the juice and with the preparation before precipitation with the acid are given. In all cases, the supernatant liquid from the acid precipitate showed very little or no infectivity. In a few cases, the acid precipitate was suspended in buffer solution (pH7) and used for inoculation. In all cases, they were as infectious as the preparations before precipitation.

(See Table 7)

The preparations from the different species were much alike both in appearance and composition. All contained approximately the same percentage of nitrogen calculated on the basis of total

TABLE 7.—ANALYSES OF PURIFIED VIRUS PREPARATIONS FROM VARIOUS SPECIES OF PLANTS.

Species and Variety	Plants Inoculated	Plants Diseased				Mg. N. per liter	% N. Basis of total solids	% Ash Basis of total solids
		Juice		Preparation				
		−c	+c	−c	+c			
Nicotiana Tabacum L.var. Turkish	10	10	10	9	10	24.9	15.9	1.2
N. macrophylla	10	10	10	10	10	32.4	15.9	1.2
N. paniculata	10	9	10	10	10	53.3	15.2	2.0
N. trigonophylla Dun.	10	8	10	10	10	21.7	12.9	0.6
N. longifolia	10	9	8	10	10	20.6	16.7	0.6
Lycopersicon pimpinellifolium Mill.	10	7	10	10	10	27.3	16.1	0.6
Lycopersicon esculentum var. Golden Pear	10	10	10	10	10	21.7	15.2	2.2

solids present. This percentage, about 16 per cent, is not far from that of simple proteins. The percentage of ash was very low, much lower than that of preparations obtained by any previous method.

In the case of other species, only the nitrogen content and infectivity were determined.

TABLE 8.—NITROGEN CONTENT AND INFECTIVITY OF PURIFIED VIRUS PREPARATIONS FROM SEVERAL SPECIES OF PLANTS

Species and Variety	Plants Diseased (10 Plants Inoculated)				Mgs. N per liter
	Juice		Preparation		
	−c	+c	−c	+c	
Nicotiana suaveolens	10	10	8	10	28.1
Nicotiana clevelandii, A. Gray	9	8	9	10	9.7
Nicotiana glauca	1	4	3	5	4.4
Nicandra physalodes	7	8	10	10	21.3
Physalis perriviana	9	10	6	9	22.0
Physalis angulata	1	3	2	6	1.8
Hyosryamus niger	9	9	10	10	27.0
Solanum melongena, var. Peking Green	10	10	10	10	7.5
Solanum tuberosum, var. Triumph	1	1	6	10	4.7
Petunia, var. General Dobbs	7	5	9	10	13.3
Spinacia oleracea, var. King of Denmark	1	1	1	6	0.9
Capsicum frutescens var. Harris Early Giant	2	4	10	10	14.3

Precipitates from *N. Tabacum, Lycopersicum esculentum* var. Golden Queen, and *Capsicum fructescens* var. Pimiento were tested by the biuret and xanthoproteic tests. All gave positive tests.

Nitrogen was present in all cases. In some cases, it was very low but the infectivity was also low. Similar preparations from juice of healthy plants of *N. Tabacum,* var Turkish contained 1.1 mg. of nitrogen per liter.

DISCUSSION

The tobacco mosaic virus is definitely associated with nitrogenous material in all preparations that have been obtained from many species of plants. A precipitate having all the properties of a simple protein and that is infectious has been isolated from several species. The precipitates contained about 16 per cent nitrogen, were very low in ash content and were precipitated by means of dilute acid. Those that were tested gave positive protein tests.

The view that the virus is a simple protein is supported by the action of proteoclastic enzymes on the virus. Pepsin inactivates the virus. This enzyme attacks a large number of simple proteins but not the lower degradation products, the peptones and polypeptides. Trypsin does not digset the virus but apparently is able to combine with it in some manner. This might possibly be a step preliminary to hydrolysis while the conditions necessary for completion are lacking. This may be due to the nature of the protein or to the lack of the proper activator. Several activators of trypsin were investigated. None of these seemed to promote hydrolysis of the virus. Trypsin is very effective in the digestion of proteoses, peptones and some polypeptides as well as many simple proteins. Papain (free of HCN) inactivates the virus[11]. The presence of hydrocyanic acid is reported as being essential for papain to digest the lower protein derivatives[26] while papain free of HCN exhibits tryptic action in some cases.

SUMMARY AND CONCLUSIONS

1. Data were obtained that indicated inactivation of the virus by trypsin is due to adsorption and not to the action of the enzyme on the host plant. Inactivation was greatest when conditions were most favorable to adsorption.

2. Pepsin inactivates the virus at pH3. Controls are not inactivated at pH3 as shown by inoculation into *N. Tabacum.*

3. Pesin inactivates the virus slowly, causing a gradual reduction in infectivity.

4. A method of purification is described which greatly reduces the ash content of the final preparation. These preparations are believed to be as pure or purer than previously described ones.

5. Purified preparations from several species of plants all contained approximately 16 per cent nitrogen and 1 per cent ash when calculated on the basis of total solids present.

6. Purified virus preparations from 19 species of plants, varying widely in nature, contained nitrogen. The nitrogen content was correlated with the infectivity.

7. The virus is either a simple protein or is closely associated with simple proteins.

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